SPECIFICATION

BACTERIUM PRODUCING L-GLUTAMIC ACID AND METHOD FOR PRODUCING L-GLUTAMIC ACID

Background of the Invention

Field of the Invention

The present invention relates to a novel L-glutamic acid producing bacterium and a method for producing L-glutamic acid by fermentation utilizing it. L-glutamic acid is an important amino acid as foodstuffs, drugs and so forth.

Description of the Related Art

Conventionally, L-glutamic acid is mainly produced by fermentative methods using so-called L-glutamic acid producing coryneform bacteria belonging to the genus *Brevibacterium, Corynebacterium* or *Microbacterium*, or mutant strains thereof (Amino Acid Fermentation, pp.195-215, Gakkai Shuppan Center, 1986).

It is known that, in the production of L-glutamic acid by fermentation, trehalose is also produced as a secondary product. Therefore, techniques have been developed for decomposing or metabolizing the produced trehalose. Such techniques include the method of producing an amino acid by fermentation using a

coryneform bacterium in which proliferation ability on trehalose is induced (Japanese Patent Laid-open (Kokai) No. 5-276935) and the method of producing amino acid by fermentation using a coryneform bacterium in which a gene coding for trehalose catabolic enzyme is amplified (Korean Patent Publication (B1) No. 165836). However, it is not known how to suppress the formation of trehalose itself in an L-glutamic acid producing bacterium.

In *Escherichia coli*, the synthesis of trehalose is catalyzed by trehalose-6-phosphate synthase. This enzyme is known to be encoded by *otsA* gene. Further, it has been also reported that an *otsA* gene-disrupted strain of *Escherichia coli* can scarcely grow in a hyperosmotic medium (H.M. Glaever, *et al*., *J. Bacteriol*., *170*(6), 2841-2849 (1998)). However, the relationship between disruption of *otsA* gene and production of substances has not been known.

On the other hand, although the *treY* gene is known for *Brevibacterium helvolum* among bacteria belonging to the genus *Brevibacterium* bacteria, any *otsA* gene is not known for them. As for bacteria belonging to the genus *Mycobacterium* bacteria, there is known a pathway via a reaction catalyzed by a product encoded by *treS* gene (trehalose synthase (TreS)), which gene is different from the *otsA* gene and *treY* gene, as a gene coding for a enzyme in trehalose biosynthesis pathway (De Smet K.A.,

et al., Microbiology, *146* (1), 199-208 (2000)). However, this pathway utilizes maltose as a substrate and does not relate to usual L-glutamic acid fermentation that utilizes glucose, fructose or sucrose as a starting material.

SUMMARY OF THE INVENTION

An object of the present invention is to improve production efficiency of L-glutamic acid in L-glutamic acid production by fermentation using coryneform bacteria through suppression of the production of trehalose as a secondary product.

The inventors of the present invention assiduously studied in order to achieve the aforementioned object. As a result, they found that bacterium belonging to the genus *Brevibacterium* contained *otsA* gene and *treY* gene like *Mycobacterium tuberculosis*, and the production efficiency of L-glutamic acid was improved by disrupting at least one of these genes. Thus, they accomplished the present invention.

That is, the present invention provides the followings.

(1) A coryneform bacterium having L-glutamic acid producing ability, wherein trehalose synthesis ability is decreased or deleted in the bacterium.

(2) The coryneform bacteria according to (1),

wherein the trehalose synthesis ability is decreased or deleted by introducing a mutation into a chromosomal gene coding for an enzyme in a trehalose synthesis pathway or disrupting the gene.

(3) The coryneform bacteria according to (2), wherein the gene coding for the enzyme in trehalose synthesis pathway consists of a gene coding for trehalose-6-phosphate synthase, a gene coding for maltooligosyltrehalose synthase, or both of these genes.

(4) The coryneform bacteria according to (3), wherein the gene coding for trehalose-6-phosphate synthase codes for the amino acid sequence of SEQ ID NO: 30, and the gene coding for maltooligosyltrehalose synthase codes for the amino acid sequence of SEQ ID NO: 32.

(5) A method for producing L-glutamic acid comprising culturing a coryneform bacterium according to any one of (1) to (4) in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium.

(6) A DNA coding for a protein defined in the following (A) or (B):

(A) a protein having the amino acid sequence of SEQ ID NO: 30,

(B) a protein having an amino acid sequence of SEQ ID NO: 30 including substitution, deletion, insertion or addition of one or several amino acid residues and

having trehalose-6-phosphate synthase activity.

(7) A DNA according to (6), which is a DNA defined in the following (a) or (b):

(a) a DNA containing a nucleotide sequence comprising at least the residues of nucleotide numbers 484-1938 in the nucleotide sequence of SEQ ID NO: 29,

(b) a DNA hybridizable with a nucleotide sequence comprising at least the residues of nucleotide numbers 484-1938 in the nucleotide sequence of SEQ ID NO: 29 under a stringent condition, showing homology of 55% or more to the foregoing nucleotide sequence, and coding for a protein having trehalose-6-phosphate synthase activity.

(8) A DNA coding for a protein defined in the following (A) or (B):

(A) a protein having the amino acid sequence of SEQ ID NO: 32,

(B) a protein having an amino acid sequence of SEQ ID NO: 32 including substitution, deletion, insertion or addition of one or several amino acid residues and having maltooligosyltrehalose synthase activity.

(9) A DNA according to (8), which is a DNA defined in the following (a) or (b):

(a) a DNA containing a nucleotide sequence comprising at least the residues of nucleotide numbers 82-2514 in the nucleotide sequence of SEQ ID NO: 31,

(b) a DNA hybridizable with a nucleotide sequence

comprising at least the residues of nucleotide numbers 82-2514 in the nucleotide sequence of SEQ ID NO: 31 under a stringent condition, showing homology of 60% or more to the foregoing nucleotide sequence, and coding for a protein having maltooligosyltrehalose synthase activity.

The trehalose-6-phosphate synthase activity means an activity to catalyze a reaction in which α,α-trehalose-6-phosphate and UDP are produced from UDP-glucose and glucose-6-phosphate, and the maltooligosyltrehalose synthase activity means an activity to catalyze a reaction in which maltotriosyltrehalose is produced from maltopentose.

According to the present invention, production efficiency of L-glutamic acid in L-glutamic acid production by fermentation using coryneform bacteria can be improved through inhibition of the production of trehalose as a secondary product.

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Preferred ~~Preferreed~~ Embodiments of the Invention

Hereafter, the present invention will be explained in detail.

The coryneform bacterium of the present invention is a coryneform bacterium having L-glutamic acid producing ability, in which trehalose synthesis ability is decreased or deleted.

The coryneform bacteria referred to in the present invention include the group of microorganisms defined in Bergey's Manual of Determinative Bacteriology, 8th edition, p.599 (1974), which are aerobic Gram-positive rods having no acid resistance and no spore-forming ability aerobic. They have hitherto been classified into the genus *Brevibacterium*, but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, *41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* or *Microbacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are mentioned below.

Corynebacterium acetoacidophilum
Corynebacterium acetoglutamicum
Corynebacterium alkanolyticum
Corynebacterium callunae
Corynebacterium glutamicum
Corynebacterium lilium (*Corynebacterium glutamicum*)
Corynebacterium melassecola
Corynebacterium thermoaminogenes
Corynebacterium herculis
Brevibacterium divaricatum (*Corynebacterium glutamicum*)
Brevibacterium flavum (*Corynebacterium glutamicum*)
Brevibacterium immariophilum
Brevibacterium lactofermentum (*Corynebacterium*

glutamicum)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium ammoniagenes (*Corynebacterium ammoniagenes*)

Brevibacterium album

Brevibacterium cerium

Microbacterium ammoniaphilum

Specifically, the following strains can be exemplified.

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium alkanolyticum ATCC21511

Corynebacterium callunae ATCC 15991

Corynebacterium glutamicum ATCC 13020, 13032, 13060

Corynebacterium lilium (*Corynebacterium glutamicum*) ATCC 15990

Corynebacterium melassecola ATCC 17965

Corynebacterium thermoaminogenes AJ12340 (FERM BP-1539)

Corynebacterium herculis ATCC13868

Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC 14020

Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC 13826, ATCC 14067

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC 13665, ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Brevibacterium ammoniagenes (*Corynebacterium ammoniagenes*) ATCC 6871

Brevibacterium album ATCC 15111

Brevibacterium cerium ATCC 15112

Microbacterium ammoniaphilum ATCC 15354

The trehalose synthesis ability of such coryneform bacteria as mentioned above can be decreased or deleted by mutagenizing or disrupting a gene coding for an enzyme in trehalose synthesis pathway using mutagenesis treatment or genetic recombination technique. Such a mutation may be a mutation that suppresses transcription or translation of the gene coding for the enzyme in trehalose synthesis pathway, or a mutation that causes elimination or decrease of an enzyme in trehalose systhesis pathway. The enzyme in trehalose systhesis pathway may be exemplified by, for example, trehalose-6-phosphate synthase, maltooligosyltrehalose synthases, or both of these.

The disruption of a gene coding for an enzyme in trehalose systhesis pathway can be performed by gene substitution utilizing homologous recombination. A gene

on a chromosome of a coryneform bacterium can be disrupted by transforming the coryneform bacterium with DNA containing a gene coding for an enzyme in trehalose systhesis pathway modified so that a part thereof should be deleted and hence the enzyme in trehalose systhesis pathway should not normally function (deletion type gene), and allowing recombination between the deletion type gene and a normal gene on the chromosome. Such gene disruption by homologous recombination has already been established. To this end, there can be mentioned a method utilizing a linear DNA or a cyclic DNA that does not replicate in coryneform bacteria and a method utilizing a plasmid containing a temperature sensitive replication origin. However, a method utilizing a cyclic DNA that does not replicate in coryneform bacteria or a plasmid containing a temperature sensitive replication origin is preferred.

The gene coding for an enzyme in trehalose systhesis pathway may be exemplified by, for example, the *otsA* gene or *treY* gene, or it may consist of both of these. Since the nucleotide sequences of the *otsA* gene and *treY* gene of *Brevibacterium lactofermentum* and flanking regions thereof have been elucidated by the present invention, those genes can be easily obtained by preparing primers based on the sequences and performing PCR (polymerase chain reaction, see White, T.J. *et al.*, *Trends Genet.*, *5*, 185 (1989)) using the primers and

chromosomal DNA of *Brevibacterium lactofermentum* as a template.

The nucleotide sequence comprising the *otsA* gene and the nucleotide sequence comprising the *treY* gene of *Brevibacterium lactofermentum* obtained in the examples described later are shown in SEQ ID NOS: 29 and 31, respectively. Further, the amino acid sequences encoded by these nucleotide sequences are shown in SEQ ID NOS: 30 and 32, respectively.

The *otsA* gene and *treY* gene each may be one coding for a protein including substitution, deletion, insertion or addition of one or several amino acids at one or a plurality of positions, provided that the activity of trehalose-6-phosphate synthase or maltooligosyltrehalose synthase encoded thereby is not deteriorated. While the number of "several" amino acids differs depending on positions or types of amino acid residues in the three-dimensional structure of the protein, it is preferably 1-40, more preferably 1-20, further preferably 1-10.

A DNA coding for the substantially same protein as trehalose-6-phosphate synthase or maltooligosyltrehalose synthase described above can be obtained by, for example, modifying each of the nucleotide sequences by, for example, the site-directed mutagenesis method so that one or more amino acid residues at a specified site should involve substitution, deletion, insertion,

addition or inversion. Such a DNA modified as described above may also be obtained by a conventionally known mutation treatment. The mutation treatment includes a method of treating DNA coding for trehalose-6-phosphate synthase or maltooligosyltrehalose *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia* harboring a DNA coding for trehalose-6-phosphate synthase or maltooligosyltrehalose with ultraviolet irradiation or a mutating agent usually used for mutation treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid.

The substitution, deletion, insertion, addition, or inversion of nucleotide as described above also includes a naturally occurring mutant or variant on the basis of, for example, individual difference or difference in species or genus of microorganisms that harbor trehalose-6-phosphate synthase or maltooligosyltrehalose.

A DNA coding for the substantially same protein as trehalose-6-phosphate synthase or maltooligosyltrehalose synthase described above can be obtained by expressing such a DNA having a mutation as described above in a suitable cell, and examining the trehalose-6-phosphate synthase activity or maltooligosyltrehalose synthase activity of the expression product.

A DNA coding for substantially the same protein as

trehalose-6-phosphate synthase can also be obtained by isolating a DNA hybridizable with a DNA having, for example, a nucleotide sequence corresponding to nucleotide numbers of 484-1938 of the nucleotide sequence shown in SEQ ID NO: 29 or a probe that can be prepared from the nucleotide sequence under a stringent condition, showing homology of 55% or more, preferably 65% or more, more preferably 75% or more, to the foregoing nucleotide sequence, and having trehalose-6-phosphate synthase activity from a DNA coding for trehalose-6-phosphate synthase having a mutation or from a cell harboring it. Similarly, a DNA coding for substantially the same protein as maltooligosyltrehalose synthase can also be obtained by isolating a DNA hybridizable with a DNA having, for example, a nucleotide sequence corresponding to nucleotide numbers of 82-2514 of the nucleotide sequence shown in SEQ ID NO: 31 or a probe that can be prepared from the nucleotide sequence under a stringent condition, showing homology of 60% or more, preferably 70% or more, more preferably 80% or more, to the foregoing nucleotide sequence, and having maltooligosyltrehalose synthase activity from a DNA coding for maltooligosyltrehalose synthase having a mutation or from a cell harboring it.

The "stringent condition" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is

difficult to clearly express this condition by using any numerical value. However, for example, the stringent condition includes a condition under which DNA's having high homology, for example, DNA's having homology of not less than 55%, preferably not less than 60%, are hybridized with each other, and DNA's having homology lower than the above level are not hybridized with each other. Alternatively, the stringent condition is exemplified by a condition under which DNA's are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 1 x SSC, 0.1 % SDS, preferably 0.1 x SSC, 0.1 % SDS, at 60°C.

As the probe, a partial sequence of each gene can also be used. Such a probe can be produced by PCR using oligonucleotides produced based on the nucleotide sequence of each gene as primers and a DNA fragment containing each gene as a template. When a DNA fragment in a length of about 300 bp is used as the probe, the washing conditions for the hybridization may consists of 50°C, 2 x SSC and 0.1% SDS.

Genes hybridizable under such conditions as described above include those having a stop codon generated in a coding region of the genes, and those having no activity due to mutation of active center. However, such mutants can be easily removed by ligating each of the genes with a commercially available

expression vector, and measuring trehalose-6-phosphate synthase activity or maltooligosyltrehalose synthase activity.

When an *otsA* gene or *treY* gene is used for the disruption of these genes on chromosomes of coryneform bacteria, the encoded trehalose-6-phosphate synthase or maltooligosyltrehalose synthase are not required to have their activities. Further, the *otsA* gene or *treY* gene used for the gene disruption may be a gene derived from another microorganism, so long as they can undergo homologous recombination with these genes of coryneform bacteria. For example, an *otsA* gene of bacterium belonging to the genus *Escherichia* or *Mycobacterium*, *treY* gene of bacterium belonging to the genus *Arthrobacter*, *Brevibacterium helvolum*, or bacterium belonging to the genus *Rhizobium* can be mentioned.

A deletion type gene of the *otsA* gene or *treY* gene can be prepared by excising a certain region with restriction enzyme(s) from a DNA fragment containing one of these genes or a part of them to delete at least a part of coding region or an expression regulatory sequence such as promoter.

Further, a deletion type gene can also be obtained by performing PCR using primers designed so that a part of gene should be deleted. Furthermore, a deletion type gene may be one obtained by single nucleotide mutation, for example, a frame shift mutation.

Gene disruption of the *otsA* gene will be explained hereafter. Gene disruption of the *treY* gene can be performed similarly.

An *otsA* gene on a host chromosome can be replaced with a deletion type *otsA* gene as follows. That is, a deletion type *otsA* gene and a marker gene for resistance to a drug, such as kanamycin, chloramphenicol, tetracycline and streptomycin, are inserted into a plasmid that cannot autonomously replicate in coryneform bacteria to prepare a recombinant DNA. A coryneform bacterium can be transformed with the recombinant DNA, and the transformant strain can be cultured in a medium containing the drug to obtain a transformant strain in which the recombinant DNA was introduced into chromosomal DNA. Alternatively, such a transformant strain can be obtained by using a temperature sensitive plasmid as the plasmid, and culturing the transformants at a temperature at which the temperature sensitive plasmid cannot replicate.

In a strain in which the recombinant DNA is incorporated into a chromosome as described above, the recombinant DNA causes recombination with an *otsA* gene sequence that originally exists on the chromosome, and two of fused genes comprising the chromosomal *otsA* gene and the deletion type *otsA* gene are inserted into the chromosome so that other portions of the recombinant DNA (vector portion and drug resistance marker gene) should

be interposed between them.

Then, in order to leave only the deletion type *otsA* gene on the chromosomal DNA, one copy of the *otsA* gene is eliminated from the chromosomal DNA together with the vector portion (including the drug resistance marker gene) by recombination of two of the *otsA* genes. In that case, the normal *otsA* gene is left on the chromosomal DNA and the deletion type *otsA* gene is excised, or conversely, the deletion type *otsA* gene is left on the chromosomal DNA and the normal *otsA* gene is excised. It can be confirmed which type of the gene is left on the chromosomal DNA by investigating structure of the *otsA* gene on the chromosome by PCR, hybridization or the like.

The coryneform bacterium used for the present invention may have enhanced activity of an enzyme that catalyzes the biosynthesis of L-glutamic acid in addition to the deletion or decrease of trehalose synthesis ability. Examples of the enzyme that catalyzes the biosynthesis of L-glutamic acid include glutamate dehydrogenase, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase, pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose

bisphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and so forth.

Further, in the coryneform bacterium used for the present invention, an enzyme that catalyzes a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid may be declined or made deficient. Examples of such an enzyme include α-ketoglutarate dehydrogenase, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroximate synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, L-glutamate decarboxylase, 1-pyrroline dehydrogenase and so forth.

Furthermore, by introducing a temperature sensitive mutation for a biotin activity inhibiting substance such as surface active agents into a coryneform bacterium having L-glutamic acid producing ability, the bacterium becomes to be able to produce L-glutamic acid in a medium containing an excessive amount of biotin in the absence of a biotin activity inhibiting substance (see WO96/06180). As such a coryneform bacterium, the *Brevibacterium lactofermentum* AJ13029 strain disclosed in WO96/06180 can be mentioned. The AJ13029 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently, the independent administrative corporation, National Institute of

Advanced Industrial Science and Technology, International Patent Organism Depositary (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-5466) on September 2, 1994, and received an accession number of FERM P-14501. Then, it was transferred to an international deposit under the provisions of the Budapest Treaty on August 1, 1995, and received an accession number of FERM BP-5189.

When a coryneform bacterium having L-glutamic acid producing ability, in which trehalose synthesis ability is decreased or deleted, is cultured in a suitable medium, L-glutamic acid is accumulated in the medium.

The medium used for producing L-glutamic acid is a usual medium that contains a carbon source, a nitrogen source, inorganic ions and other organic trace nutrients as required. As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, sucrose, maltose, blackstrap molasses and starch hydrolysate; alcohols such as ethanol and inositol; or organic acids such as acetic acid, fumaric acid, citric acid and succinic acid.

As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium phosphate and ammonium acetate, ammonia, organic nitrogen such as peptone, meat extract, yeast extract, corn steep liquor and soybean hydrolysate, ammonia gas, aqueous ammonia

and so forth.

As the inorganic ions (or sources thereof), added is a small amount of potassium phosphate, magnesium sulfate, iron ions, manganese ions and so forth. As for the organic trace nutrients, it is desirable to add required substances such as vitamin B_1, yeast extract and so forth in a suitable amount as required.

The culture is preferably performed under an aerobic condition performed by shaking, stirring for aeration or the like for 16 to 72 hours. The culture temperature is controlled to be at 30°C to 45°C, and pH is controlled to be 5 to 9 during the culture. For such adjustment of pH, inorganic or organic acidic or alkaline substances, ammonia gas and so forth can be used.

Collection of L-glutamic acid from fermentation broth can be performed by, for example, methods utilizing ion exchange resins, crystallization and so forth. Specifically, L-glutamic acid can be adsorbed on an anion exchange resin and isolated from it, or crystallized by neutralization.

EXAMPLES

Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Construction of *otsA* gene-disrupted strain of *Brevibacterium lactofermentum*

<1> Cloning of *otsA* gene

Since *otsA* gene of *Brevibacterium lactofermentum* was not known, it was obtained by utilizing a nucleotide sequence of *otsA* gene of another microorganism for reference. The *otsA* genes of *Escherichia* and *Mycobacterium* had been hitherto elucidated for their entire nucleotide sequences (Kaasen I., *et al.*, *Gene*, *145* (1), 9-15 (1994); De Smet K.A., *et al.*, *Microbiology*, *146* (1), 199-208 (2000)). Therefore, referring to an amino acid sequence deduced from these nucleotide sequences, DNA primers P1 (SEQ ID NO: 1) and P2 (SEQ ID NO: 2) for PCR were synthesized first. The DNA primers P1 and P2 corresponded to the regions of the nucleotide numbers of 1894-1913 and 2531-2549 of the nucleotide sequence of the *otsA* gene of *Escherichia coli* (GenBank accession X69160), respectively. They also corresponded to the regions of the nucleotide numbers 40499-40518 and 41166-41184 of the *otsA* gene of *Mycobacterium tuberculosis* (GenBank accession Z95390), respectively.

Then, PCR was performed by using the primers P1 and P2 and chromosomal DNA of *Brevibacterium lactofermentum* ATCC 13869 as a template with a cycle consisting of reactions at 94°C for 0.5 minute, 50°C for 0.5 minute and 72°C for 4 minutes, which was repeated

for 30 cycles. As a result, a substantially single kind of amplified fragment of about 0.6 kbp was obtained. This amplified fragment was cloned into a plasmid vector pCR2.1 by using "Original TA Cloning Kit" produced by Invitrogen to obtain pCotsA. Then, the nucleotide sequence of the cloned fragment was determined.

Based on the nucleotide sequence of the partial fragment of *otsA* gene obtained as described above, DNA primers P10 (SEQ ID NO: 8) and P12 (SEQ ID NO: 10) were newly synthesized, and unknown regions flanking to the partial fragment was amplified by "inverse PCR" (Triglia, T. *et al.*, *Nucleic Acids Res.*, *16*, 81-86 (1988); Ochman H., *et al.*, *Genetics*, *120*, 621-623 (1988)). The chromosomal DNA of *Brevibacterium lactofermentum* ATCC 13869 was digested with a restriction enzyme *Bam*HI, *Bgl*II, *Cla*I, *Hin*dIII, *Kpn*I, *Mlu*I, *Mun*L, *Sal*I or *Xho*I, and self-ligated by using T4 DNA ligase (Takara Shuzo). By using resultant DNA as a template and the DNA primers P10 and P12, PCR was performed with a cycle consisting of reactions at 94°C for 0.5 minute, 55°C for 1 minute and 72°C for 4 minutes, which was repeated for 30 cycles. As a result, when *Cla*I or *Bgl*II was used as the restriction enzyme, an amplified fragment of 4 kbp was obtained for each case. The nucleotide sequences of these amplified fragments were directly determined by using the DNA primers P5 to P9 (SEQ ID NOS: 3-7) and P11 to P15 (SEQ ID NOS: 9-13). Thus, the entire nucleotide

sequence of *otsA* gene of *Brevibacterium lactofermentum* ATCC 13869 was determined as shown in SEQ ID NO: 29. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NOS: 29 and 30.

When homology of the sequence of the aforementioned *otsA* gene was determined with respect to the *otsA* gene of *Escherichia coli* (GenBank accession X69160) and the *otsA* gene of *Mycobacterium tuberculosis* (GenBank accession Z95390), the nucleotide sequence showed homologies of 46.3% and 55.9%, respectively, and the amino acid sequence showed homologies of 30.9% and 51.7%, respectively. The homologies were calculated by using software, "GENETIX-WIN" (Software Development), based on the Lipman-Person method (*Science*, *227*, 1435-1441 (1985)).

<2> Preparation of plasmid for *otsA* gene disruption

In order to examine presence or absence of improvement effect in L-glutamic acid productivity by disruption of a gene coding for an enzyme in trehalose biosysthesis pathway in coryneform bacteria, a plasmid for *otsA* gene disruption was produced. A plasmid for *otsA* gene disruption was produced as follows. PCR was performed by using the plasmid pCotsA previously constructed in the cloning of the *otsA* gene as a template and the primers P29 (SEQ ID NO: 33) and P30 (SEQ ID NO: 34) comprising *Cla*I site with a cycle

consisting of reactions at 94°C for 0.5 minute, 55°C for 0.5 minute and 72°C for 8 minutes, which was repeated for 30 cycles. The amplified fragment was digested with *Cla*I, blunt-ended by using T4 DNA polymerase (Takara Shuzo), and self-ligated by using T4 ligase (Takara Shuzo) to construct a plasmid pCotsAC containing the *otsA* gene having a frame shift mutation (1258-1300th nucleotides of SEQ ID NO: 29 were deleted) at an approximately central part thereof.

<3> Preparation of *otsA* gene-disrupted strain

By using the plasmid pCotsAC for gene disruption, a L-glutamic acid producing bacterium, *Brevibacterium lactofermentum* ATCC 13869, was transformed by the electric pulse method, and transformants were selected as to the ability to grow in CM2B medium containing 20 mg/L of kanamycin. Because the plasmid pCotsAC for *otsA* gene disruption did not have a replication origin that could function in *Brevibacterium lactofermentum*, resultant transformants obtained by using the plasmid suffered homologous recombination occurred between the *otsA* genes on the chromosome of *Brevibacterium lactofermentum* and the plasmid pCotsAC for gene disruption. From the homologous recombinant strains obtained as described above, strains in which the vector portion of the plasmid pCotsAC for gene disruption was eliminated due to re-occurrence of homologous

recombination were selected based on acquired kanamycin sensitivity as a marker.

From the strains obtained as described above, a strain introduced with the desired frame shift mutation was selected. Selection of such a strain was performed by PCR using chromosomal DNA extracted from a strain that became kanamycin sensitive as a template and the DNA primers P8 (SEQ ID NO: 14) and P13 (SEQ ID NO: 11) with a cycle consisting of reactions at 94°C for 0.5 minute, 55°C for 0.5 minute and 72°C for 1 minutes, which was repeated for 30 cycles, and sequencing of the obtained amplified fragment using the DNA primer P8 to confirm disfunction of the *otsA* gene due to introduction of frame shift mutation. The strain obtained as described above was designated as ΔOA strain.

<u>Example 2: Construction of *treY* gene-disrupted strain</u>

<1> Cloning of *treY* gene

Since *treY* gene of *Brevibacterium lactofermentum* was not known, it was obtained by using nucleotide sequences of *treY* genes of the other microorganisms for reference. The nucleotide sequences of *treY* genes were hitherto elucidated for the genera *Arthrobacter*, *Brevibacterium* and *Rhizobium* (Maruta K., *et al.*, *Biochim. Biophys. Acta*, *1289* (1), 10-13 (1996); Genbank accession AF039919; Maruta K., *et al.*, *Biosci. Biotechnol. Biochem.*, *60* (4), 717-720 (1996)). Therefore, referring

to an amino acid sequence deduced from these nucleotide sequences, the PCR DNA primers P3 (SEQ ID NO: 14) and P4 (SEQ ID NO: 15) were synthesized first. The DNA primers P3 and P4 correspond to the regions of the nucleotide numbers of 975-992 and 2565-2584 of the nucleotide sequence of the *treY* gene of *Arthrobacter* species (GenBank accession D63343), respectively. Further, they correspond to the regions of the nucleotide numbers 893-910 and 2486-2505 of the *treY* gene of *Brevibacterium helvolum* (GenBank accession AF039919), respectively. Furthermore, they correspond to the regions of the nucleotide numbers of 862-879 and 2452-2471 of *treY* gene of *Rhizobium* species (GenBank accession D78001).

Then, PCR was performed by using the primers P3 and P4 and chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as a template with a cycle consisting of reactions at 94°C for 0.5 minute, 55°C for 0.5 minute and 72°C for 2 minutes, which was repeated for 30 cycles. As a result, a substantially single kind of an amplified fragment of about 1.6 kbp was obtained. This amplified fragment was cloned into a plasmid vector pCR2.1 by using "Original TA Cloning Kit" produced by Invitrogen. Then, the nucleotide sequence was determined for about 0.6 kb.

Based on the nucleotide sequence of the partial fragment of *treY* gene obtained as described above, the DNA primers P16 (SEQ ID NO: 16) and P26 (SEQ ID NO: 26)

were newly synthesized, and unknown regions flanking to the partial fragment was amplified by "inverse PCR" (Triglia, T. *et al.*, *Nucleic Acids Res.*, *16*, 81-86 (1988); Ochman H., *et al.*, *Genetics*, *120*, 621-623 (1988)). The chromosomal DNA of *Brevibacterium lactofermentum* ATCC 13869 was digested with a restriction enzyme *Bam*HI, *Hin*dIII, *Sal*I or *Xho*I, and self-ligated by using T4 DNA ligase (Takara Shuzo). By using this as a template and the DNA primers P16 and P26, PCR was performed with a cycle consisting of reactions at 94°C for 0.5 minute, 55°C for 1 minute and 72°C for 4 minutes, which was repeated for 30 cycles. As a result, when *Hin*dIII or *Sal*I was used as the restriction enzyme, an amplified fragment of 0.6 kbp or 1.5 kbp was obtained, respectively. The nucleotide sequences of these amplified fragments were directly determined by using the DNA primers P16 to P28 (SEQ ID NOS: 16-28). Thus, the entire nucleotide sequence of *treY* gene of *Brevibacterium lactofermentum* ATCC 13869 was determined as shown in SEQ ID NO: 31. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NOS: 31 and 32.

When homology of the sequence of the aforementioned *treY* gene was determined with respect to the *treY* gene of *Arthrobacter* sp. (GenBank accession D63343), *treY* gene of *Brevibacterium helvolum* (GenBank accession AF039919) and *treY* gene of *Rhizobium* sp.

(GenBank accession D78001), the nucleotide sequence showed homologies of 52.0%, 52.3% and 51.9%, respectively, and the amino acid sequence showed homologies of 40.9%, 38.5% and 39.8%, respectively. The homologies were calculated by using software, "GENETIX-WIN" (Software Development), based on the Lipman-Person method (*Science*, *227*, 1435-1441 (1985)).

<2> Preparation of plasmid for *treY* gene disruption

In order to examine presence or absence of improvement effect in L-glutamic acid productivity by disruption of the gene coding for the enzyme in trehalose biosysthesis pathway in coryneform bacteria, a plasmid for *treY* gene disruption was produced. First, PCR was performed by using the primers P17 (SEQ ID NO: 17) and P25 (SEQ ID NO: 25) and the chromosomal DNA of ATCC 13869 as a template with a cycle consisting of reactions at 94°C for 0.5 minute, 60°C for 0.5 minute and 72°C for 2 minutes, which was repeated for 30 cycles. The amplified fragment was digested with *Eco*RI and ligated to pHSG299 (Takara Shuzo) digested with *Eco*RI by using T4 DNA ligase (Takara Shuzo) to obtain a plasmid pHtreY. Further, this pHtreY was digested with *Afl*II (Takara Shuzo), blunt-ended by using T4 DNA polymerase (Takara Shuzo), and self-ligated by using T4 ligase (Takara Shuzo) to construct a plasmid pHtreYA containing the *treY* gene having a frame shift mutation (four

nucleotides were inserted after the 1145th nucleotide in the sequence of SEQ ID NO: 31) at an approximately central part thereof.

<3> Preparation of *treY* gene-disrupted strain

By using the plasmid pCtreYA for gene disruption, a L-glutamic acid producing bacterium, *Brevibacterium lactofermentum* ATCC 13869, was transformed by the electric pulse method, and transformants were selected as to the ability to grow in CM2B medium containing 20 mg/L of kanamycin. Because the plasmid pCtreYA for *treY* gene disruption does not have a replication origin that could function in *Brevibacterium lactofermentum*, the transformants obtained by using the plasmid suffered recombination occurred between the *treY* genes on the *Brevibacterium lactofermentum* chromosome and the plasmid pCtreYA for gene disruption. From the homologous recombinant strains obtained as described above, strains in which the vector portion of the plasmid pCtreYA for gene disruption was eliminated due to re-occurrence of homologous recombination were selected based on acquired kanamycin sensitivity as a marker.

From the strains obtained as described above, a strain introduced with the desired frame shift mutation was selected. Selection of such a strain was performed by PCR using the DNA primers P19 (SEQ ID NO: 19) and P25 (SEQ ID NO: 25) with a cycle consisting of reactions at

94°C for 0.5 minute, 55°C for 0.5 minute and 72°C for 1.5 minutes, which was repeated for 30 cycles, and sequencing the obtained fragment using the DNA primer P21 or P23 to confirm dysfunction of the *treY* gene due to introduction of frame shift mutation. The strain obtained as described above was designated as ΔTA strain.

Example 3: Evaluation of L-glutamic acid producing ability of ΔOA strain and ΔTA strain

The ATCC 13869 strain, ΔOA strain and ΔTA strain were each cultured for producing L-glutamic acid as follows. Each of these strains was refreshed by culturing it on a CM2B plate medium, and each refreshed strain was cultured in a medium containing 80 g of glucose, 1 g of KH_2PO_4, 0.4 g of $MgSO_4$, 30 g of $(NH_4)_2SO_4$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 15 ml of soybean hydrolysate solution, 200 μg of thiamin hydrochloride, 3 μg of biotin and 50 g of $CaCO_3$ in 1 L of pure water (adjusted to pH 8.0 with KOH) at 31.5°C. After the culture, amount of L-glutamic acid accumulated in the medium and absorbance at 620 nm of the culture broth diluted 51 times were measured. The results are shown in Table 1.

The *Brevibacterium lactofermentum* strains of which *otsA* gene or *treY* gene was disrupted showed growth in a degree similar to that of the parent strain, and in addition, increased L-glutamic acid production compared

with the parent strain.

Table 1

Strain	OD_{620} (x51)	L-Glutamic acid (g/L)	Yield (%)
ATCC 13869	0.930	40.2	48.4
ΔOA	1.063	43.8	52.8
ΔTA	0.850	45.6	54.9

(Explanation of Sequence Listing)

SEQ ID NO: 1: Primer P1 for amplification of *otsA*

SEQ ID NO: 2: Primer P2 for amplification of *otsA*

SEQ ID NO: 3: Primer P5

SEQ ID NO: 4: Primer P6

SEQ ID NO: 5: Primer P7

SEQ ID NO: 6: Primer P8

SEQ ID NO: 7: Primer P9

SEQ ID NO: 8: Primer P10

SEQ ID NO: 9: Primer P11

SEQ ID NO: 10: Primer P12

SEQ ID NO: 11: Primer P13

SEQ ID NO: 12: Primer P14

SEQ ID NO: 13: Primer P15

SEQ ID NO: 14: Primer P3 for amplification of *treY*

SEQ ID NO: 15: Primer P4 for amplification of *treY*

SEQ ID NO: 16: Primer P16

SEQ ID NO: 17: Primer P17

SEQ ID NO: 18: Primer P18

SEQ ID NO: 19: Primer P19

SEQ ID NO: 20: Primer P20

SEQ ID NO: 21: Primer P21

SEQ ID NO: 22: Primer P22

SEQ ID NO: 23: Primer P23

SEQ ID NO: 24: Primer P24

SEQ ID NO: 25: Primer P25

SEQ ID NO: 26: Primer P26

SEQ ID NO: 27: Primer P27

SEQ ID NO: 28: Primer P28

SEQ ID NO: 29: Nucleotide sequence of *otsA* gene

SEQ ID NO: 30: Amino acid sequence of OtsA

SEQ ID NO: 31: Nucleotide sequence of *treY* gene

SEQ ID NO: 32: Amino acid sequence of TreY

SEQ ID NO: 33: Primer P29

SEQ ID NO: 34: Primer P30